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Award Number: W81XWH-04-1-0542

TITLE: D2 as an Integrator of Oncogenic Stimuli in Breast Cancer

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REPORT DATE: August 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-08-2007	Final	23 JUL 2004 - 22 JUL 2007

4. TITLE AND SUBTITLE

D2 as an Integrator of Oncogenic Stimuli in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0542

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Case Western Reserve University
Cleveland, OH 44106

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This proposal focused on the role of Inhibitor of Differentiation (Id) proteins, which are transcription factors, in mammary gland development and carcinogenesis. Although Id2 regulates mammary gland growth and development and is important for myc-induced transformation of certain cell types, its role in breast cancer has not been thoroughly addressed. We have found that Id2 is upregulated in two mouse models of breast cancer: one that involves hormone-induction of carcinogenesis and the other involves overexpression of HER2/neu. In contrast, Id1 expression is reduced in both models. These data suggest that Id2 may be a common integrator of oncogenic stimuli in breast and that Id1 may suppress breast carcinogenesis. The goals of this work were to determine, using transgenic mouse models, if Id2 is necessary and sufficient for mammary gland tumorigenesis in mice. If so, this would suggest that Id2 or processes regulated by this transcription factor may serve as useful prognostic markers or therapeutic targets in breast cancer.

15. SUBJECT TERMS
HER2/Neu/erbB2, Inhibitor of Differentiation, Id1, Id2, Transgenic Mouse, Breast Cancer

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

Inhibitor of DNA Binding (Id1-4) proteins are members of the helix-loop-helix family of transcription factors that lack a DNA binding domain (1). The Id proteins bind and repress the functions of ubiquitously expressed E-proteins and have been classically associated with inhibiting E-protein driven cellular differentiation. Both Id1 and Id2 have been implicated in mammary gland development and tumorigenesis, but their specific roles in these processes remains controversial (2-5). Id2 deficient mice have a mid-pregnancy mammary gland developmental defect resulting from a decrease in epithelial cell proliferative rate and an increase in apoptotic rate (2;6). These *in vivo* data suggest an important role in regulating proliferation of mammary epithelial cells. However, expression patterns during mammary gland development and *in vitro* analyses suggest that this protein may also be an essential inducer of differentiation of the mammary epithelium. In contrast to Id2 deficient animals, mice lacking Id1 do not have a mammary gland defect, but *in vitro* studies suggest that this protein can induce proliferation of breast cancer cell lines (7-9). Furthermore, correlation studies in human breast cancer suggest that Id1 expression may be a marker for aggressive tumors (10). To assess the roles of Ids, in particular Id2, in mammary gland development and carcinogenesis, we proposed three goals in the Statement of Work. These included 1) Determining if Id2 deficiency regulates hormone- and/or Neu-induced mammary tumorigenesis. 2) Determining if elevated expression of Id2 increases mammary tumor susceptibility. 3) Identifying the mechanisms underlying transcriptional control of the Id2 gene in the mammary gland. These studies would test our **central hypothesis: Id2 integrates many oncogenic stimuli to control the initiation and growth of breast cancer.**

BODY

Using mouse models of breast cancer, we found that Id2 mRNA levels are upregulated with the development of hyperplasia and tumors (figure 1A and B). In contrast, Id1 mRNA levels were reduced with the development of hyperplasia (Figure 1C and D). The consistent changes in expression of these two genes were intriguing because the breast cancer models that were evaluated were highly distinct. One model involves luteinizing hormone overexpression in the pituitary that induces ovarian hyperstimulation and subsequent hormone-induced mammary cancer [(LH-overexpressing mice, (11)]. The second model involves HER2/neu overexpression [MMTV-c-Neu transgenic mouse, (12)]. These changes occurred in the expression pattern of the endogenous mRNAs. Thus, we hypothesized that increased Id2 expression may promote mammary gland tumorigenesis while sustained Id1 may prevent hyperplasia and tumorigenesis. The proposal was originally focused on

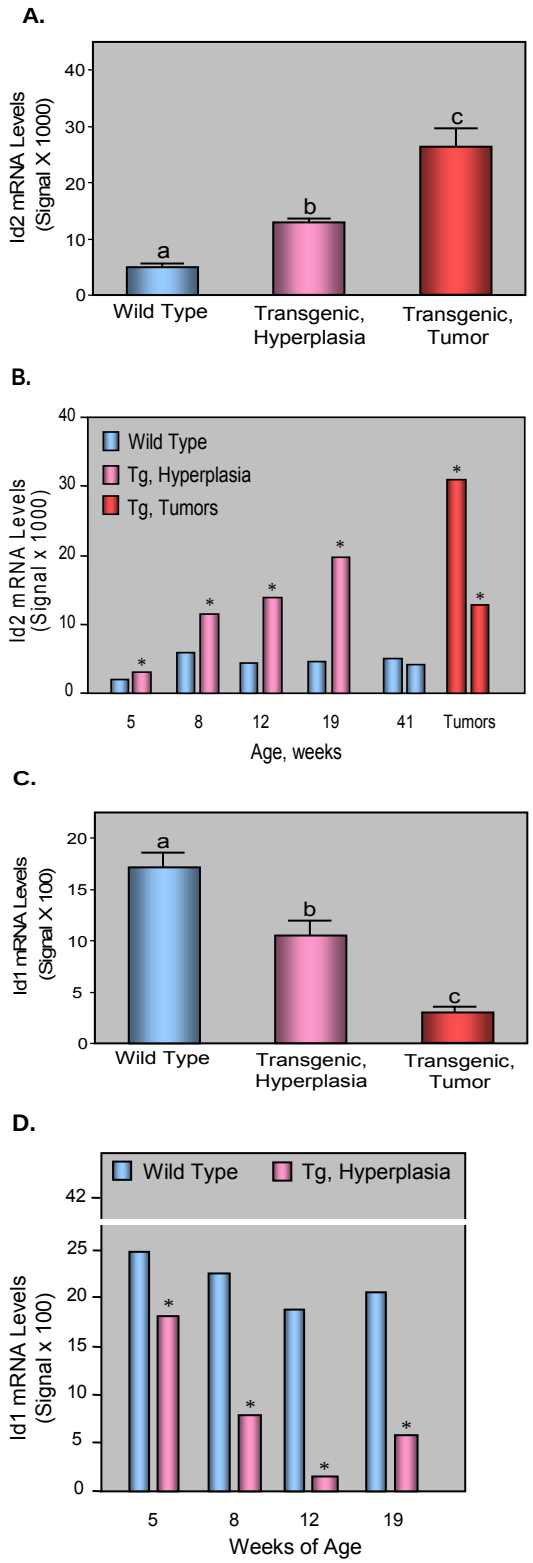


Figure 1. Id2 mRNA is progressively increased in two mouse models of breast cancer and Id1 mRNA levels are decreased in two models of hyperplasia. All mRNA evaluations were performed with Affymetrix U74Av2 gene expression microarrays. Panels A and C are data from MMTV-c-Neu mice and Panels B and D are from LH-overexpressing mice.

Id2, however, we realized that some studies should also evaluate Id1 because this would allow a more thorough assessment of the functions of these related proteins in mammary gland development and carcinogenesis.

The first aim of the proposal was focused on determining if Id2 was a NECESSARY factor for mammary tumorigenesis in mice. We had proposed using Id2 knock-out mice for this analysis. Specifically, we would assess tumorigenic rate in LH-overexpressing mice and in MMTV-c-Neu mice in the presence and absence of endogenous Id2. Id2 deficiency had previously been shown to inhibit lactational development of the mammary gland (2;6), thus the knock-outs appeared to be viable. We obtained the Id2 heterozygous null mice from Yoshifumi Yokota and began the necessary breeding paradigm to generate combined knock-out/transgenic mice. After more than one year of breeding and genotyping mice, we had obtained only one adult MMTV-c-Neu mouse that was null for the Id2 locus. This indicated that the Id2 deficient mice have a severe reduction in survival. Dr. Yokota confirmed that post-natal lethality was common in multiple genetic strains of mice with frequent losses of 80% of null animals. During this time, multiple manuscripts were published examining the requirement for Id2 in a variety of tumorigenic mouse models. All had small populations of null mice compared to a typical tumor latency analysis, supporting the limitation of collecting large groups of null animals in a variety of mouse strains. More importantly, these studies showed that Id2 was not necessary for Wnt1-induced mammary tumorigenesis (13) and myc-induced epidermal neoplasia (14) and lymphomagenesis (15). While these data did not directly assess hormone or HER2/Neu-induced tumorigenesis, it suggested that Id2 may not be necessary for many mechanisms of tumorigenesis. As a result of the limitations in collecting sufficient animals to assess changes in tumor latency and the increasing number of reports indicating that tumor development could occur in the absence of Id2, we turned our attention to determining if Id2 overexpression was SUFFICIENT to induce mammary gland tumorigenesis.

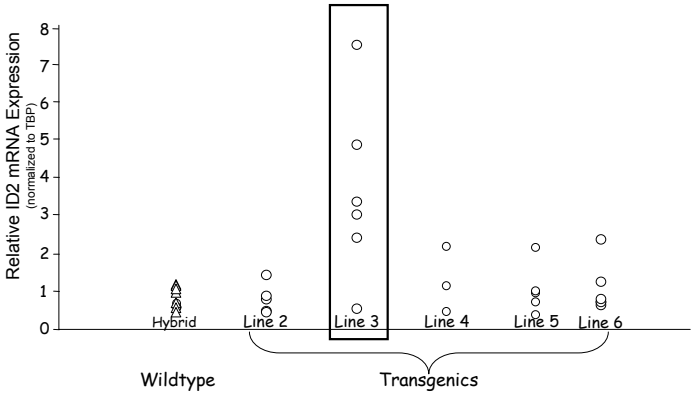


Figure 2. MMTV-Id2 transgenic line #3 overexpresses Id2 mRNA in the mammary gland. Transgenic mice were produced and the levels of Id2 mRNA evaluated in the mammary gland with quantitative real time RT-PCR. All values are expressed relative to the mRNA values for TATA-Binding Protein (TBP), an internal control. Individual points are from individual enimals. This assay detects endogenous and transgenic Id2, hence wild type mice also have detectable Id2 mRNA levels in the mammary gland. Transgenic line #3 has significantly elevated levels of Id2 in all but one mouse in this assay.

The second aim of the proposal focused on assessing whether Id2 overexpression could induce mammary tumors. The aim centered on the development of transgenic mice that overexpress Id2 in the mammary gland utilizing the MMTV promoter and determining if this induced tumors with age. While not included in the original proposal, several reports also emerged during this work that suggested that Id1 and Id2 may play contrasting roles in the mammary gland (5;10;16-18). Thus, we concluded that it was necessary to compare the effects of Id1 and Id2 overexpression in the mammary gland. Both types of mice were produced using the MMTV promoter to direct expression to the mammary gland.

To determine if MMTV-Id2 mice express the transgene in the mammary gland, we quantified mRNA levels for Id2. As shown in figure 2, we confirmed that Id2 mRNA was elevated in mammary glands of MMTV-Id2 mice from transgenic line #3. All subsequent studies were performed with this line. To assess the impact of Id2 expression on the normal mammary

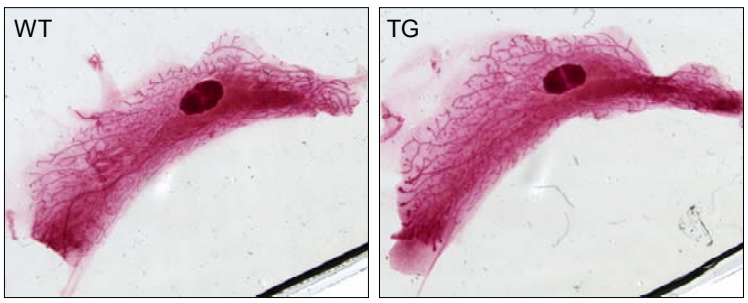


Figure 3. Whole mount analysis of mammary glands from 6 week old mice indicates that MMTV-Id2 transgenic glands do not display overt morphological differences from wild type glands. WT=wild type gland, TG =transgenic gland.

gland, we evaluated whole mounts of mammary glands from these mice. As shown in figure 3, MMTV-Id2 transgenic mice had no overt phenotype in the mammary glands. Specifically, there was no evidence of hyper- or hypo-plasia and this was consistent across various stages of the estrus cycle and with extended age. MMTV-Id2 female transgenic mice were also able to nurse litters, indicating that the mammary glands were capable of undergoing complete terminal differentiation (data not shown).

We further examined the proliferative and apoptotic rates of the mammary epithelium in Id2 transgenic females. No differences were observed in either of these parameters in virgin and pregnant mice (figure 4). Studies are currently underway to determine if apoptotic rate may be distinct between transgenic and wild type mice during post-lactational involution. To determine if Id2 may regulate tumor initiation or progression, we aged a cohort of mice to assess their rate of spontaneous tumor development. Another cohort of mice was generated for treatment with the mammary carcinogen, 7,12 dimethylbenz[a]-anthracene (DMBA). As shown in figure 5A, mice in the FVB/N genetic background that are over one year of age occasionally form a mammary tumor. The frequency of mice that formed such tumors was indistinguishable for MMTV-Id2 transgenic and wild type mice. In addition, the rates of tumor formation of DMBA treated wild type and transgenic MMTV-Id2 were not statistically different (figure 5B). These data indicate that Id2 overexpression neither induces spontaneous tumors nor accelerates DMBA-induced tumorigenesis. We next assessed the impact of Id2 overexpression on HER2/Neu-induced tumorigenesis. The rates of tumor formation were compared between bi-transgenic MMTV-Id2/MMTV-c-Neu mice and those with the MMTV-c-Neu transgene alone. Again, no significant differences in tumor latency were observed in this study (figure 6). Together, these results indicate that Id2 overexpression fails to impact the proliferative and apoptotic rates of the mammary epithelium and that it does not impact chemical carcinogen or HER2/Neu-induced tumorigenesis. These data disproved our original hypothesis regarding the ability of Id2 to modulate various forms of tumorigenesis. However, it should be noted that we can not conclude whether Id2 is required for hormone or HER2/Neu-induced tumorigenesis. Testing this possibility will require the development of a conditional Id2 deficient mouse, or the use of xenograft models of tumorigenesis. Our next approach for evaluating Id2 necessity for tumor formation and progression will involve the use of shRNA knock-downs of Id2 in breast cancer cell lines, followed by evaluation of growth in immune-compromised mice. However, this analysis extends beyond the original grant proposal.

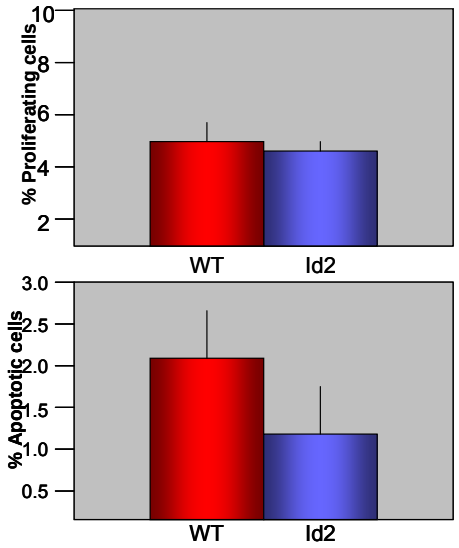


Figure 4. MMTV-Id2 transgenic mouse mammary glands do not have detectable differences in proliferation or apoptosis compared to wild type mice. WT=wild type glands, TG=transgenic glands. Shown is data collected from glands taken at pregnancy day 14 and immunostained with either phospho-histone H3 as a marker of proliferation or Apoptag Tunel analysis as an indicator of apoptosis.

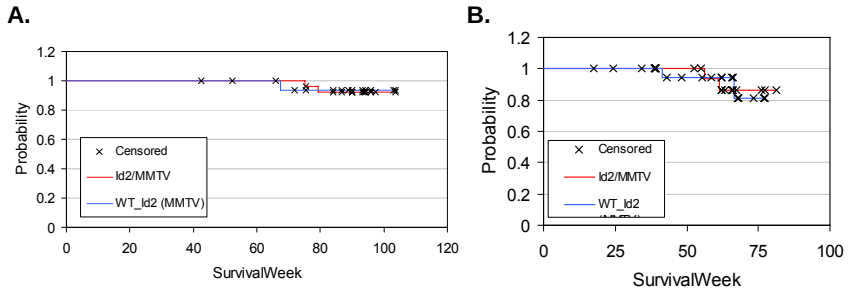
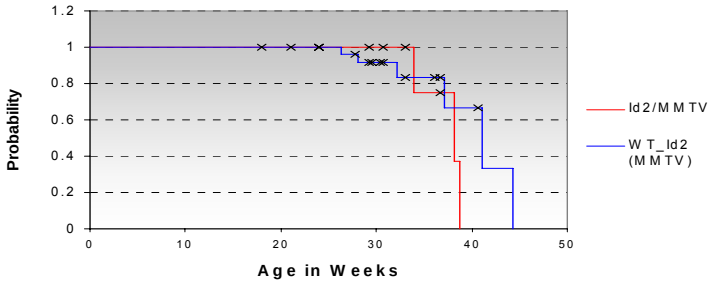


Figure 5. MMTV-Id2 have no differences in spontaneous or DMBA-induced tumor latency compared to wild type mice. Shown are Kaplan-Meier survival latency curves. **A)** Spontaneous tumor latency was examined by weekly palpation in a cohort of MMTV-Id2 and age-matched wild type littermates. This experiment was terminated when the mice reached 2 years of age. **B)** MMTV-Id2 and age matched wild type littermates were treated with two doses of 1 mg DMBA by oral gavage at 5 and 6 weeks of age and then palpated weekly for the development of mammary tumors. This experiment was terminated when the mice reached 18 months of age. Censored events are mice that died or had to be removed from the study for reasons other than development of a mammary tumor.

While several reports have suggested that Id2 might play a role in tumorigenesis (19-24), other studies indicate that Id1 may be more prominent in carcinogenesis (7-10;18). To directly compare the

tumorigenic properties of Id1 and Id2, we also produced Id1 transgenic mice. We identified one line of MMTV-Id1 transgenic mice that highly over-express Id1 mRNA in the mammary glands (figure 7). However, mammary glands from MMTV-Id1 transgenic mice do not have alterations in their ductal architecture compared to wild type animals (figure 8). This is consistent across various stages of the estrous cycle and with extended age (data not shown). We have also begun a tumor latency study with MMTV-Id1 mice. These mice were crossed with MMTV-c-Neu animals to generate MMTV-Id1/MMTV-c-Neu bi-transgenics and MMTV-c-Neu single transgenics to determine if overexpression of Id1 accelerates Neu-induced tumor development or growth. These mice are currently ~8 months of age and are being palpated weekly. If we find that Id1 overexpression accelerates tumor formation while Id2 overexpression does not, this would indicate that Id1 and Id2 play distinct roles in this process. Future studies would be aimed at determining the subset of Id target genes that are uniquely regulated by Id1.

Figure 6. Overexpression of Id2 fails to decrease the latency of HER2/Neu-induced mammary tumorigenesis.



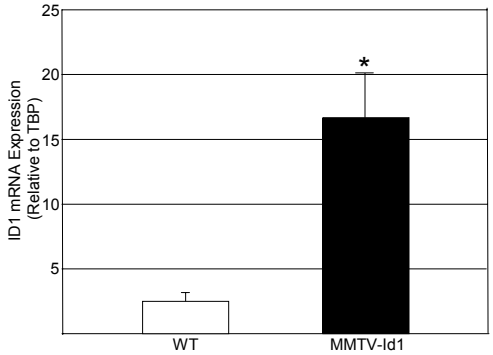


Figure 7. Id1 mRNA is over-expressed in MMTV-Id1 transgenic mouse mammary glands. Transgenic mice were produced and the levels of Id1 mRNA evaluated in the mammary gland with quantitative real time RT-PCR. Shown is one line of transgenic mice that was used for all subsequent studies. All values are expressed relative to the mRNA values for TATA-Binding Protein (TBP), an internal control. Individual points are from individual enimals. This assay detects endogenous and transgenic Id1, hence wild type mice also have detectable Id1 mRNA levels in the mammary gland. * = p<0.01

The third aim of this proposal focused on determining mechanisms by which the Id2 gene is regulated. To accomplish this goal, we used an Id2 promoter construct coupled with transient transfections. To determine if the Id2 promoter was activated by HER2/Neu signaling, MCF-7 cells were transfected and treated with Heregulin, a ligand for HER3/erbB3 and indirect activator of HER2 (figure 8). These experiments revealed that Heregulin only modestly increases activity of the Id2 promoter by approximately 50%. These studies only assess a subset of HER2 signaling, specifically that emanating from a HER2/HER3 or HER2/HER4 heterodimer and in cells whose transformation properties are not dependent upon HER2. To assess the impact of HER2 signaling in a HER2 positive breast cancer cell line, we transiently transfected BT-474 cells, which are dependent upon amplified HER2 and treated those cells with a HER2 inhibitor, AG825. Consistent with the heregulin studies, this approach revealed that the Id2 promoter is ~50% suppressed with AG825 treatment (figure 9). In both paradigms, inhibition or activation of HER2 was confirmed by western blotting for phosphorylated AKT, a direct read-out of HER2/HER3 activation (data not shown). Taken together, these results indicate that while the Id2 promoter is regulated by HER2, the extent of HER2-mediated activation of Id2 transcription

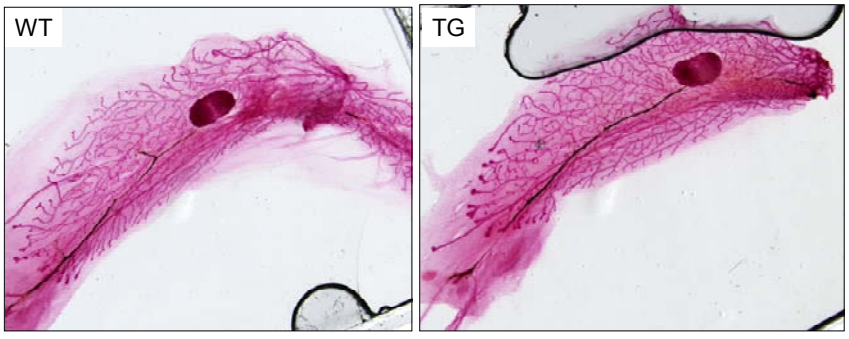


Figure 8. Whole mounts of 6-week old MMTV-Id1 mammary glands revealed no gross morphological differences from wild type glands. WT = wild type, TG = transgenic

fairly minor. Furthermore, this low level of activation would likely preclude accurate identification of *cis*-acting elements involved in this process. These data also support the hypothesis that the Id2 gene is not directly downstream of HER2. Rather, its upregulation in mammary cancer initiated by diverse stimuli may reflect a selection process that shifts cellular composition and favors the specific cell types that are capable of expressing Id2. Whether Id2 plays any role in these cell types will require direct assessment *in vitro* or with mammary-specific conditional knock-out models.

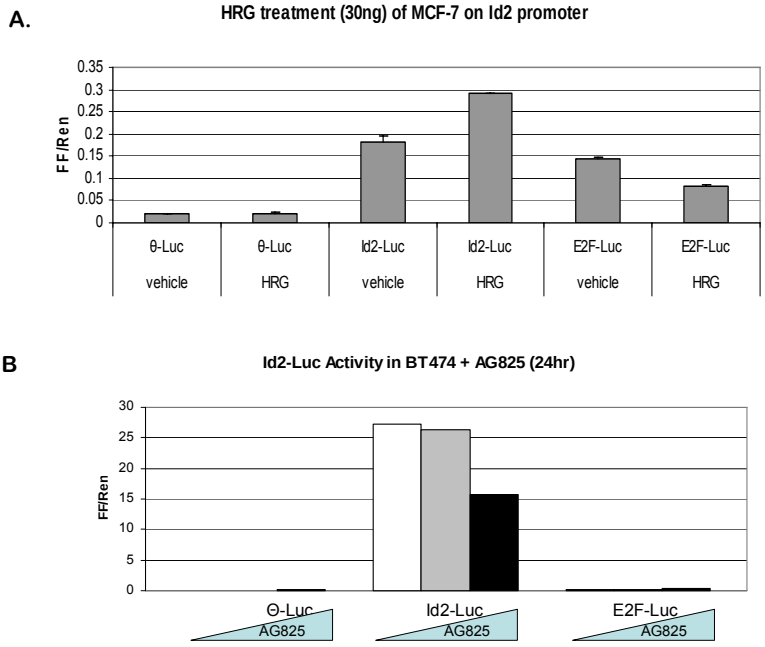


Figure 9. The Id2 promoter is only modestly regulated by HER2. A) MCF-7 cells were transiently transfected with the indicated promoters directing the expression of luciferase. All cells were also transfected with CMV-Renilla as a control for transfection efficiency. Following transfection, cells were treated with heregulin, a HER3 ligand that indirectly activates HER2 in these cells. Shown is the relative expression levels following normalization for renilla values. **B)** BT-474 cells were transiently transfected with the promoter constructs indicated and then treated with AG825, a selective HER2 inhibitor. Luciferase values are expressed relative to renilla values, the transfection efficiency control.

Recent studies have suggested that Id2 translocation to the nucleus is stimulated by RANKL. We also performed studies to determine if activation of HER2 or the EGF receptor would induce translocation of Id2 into the nucleus. This involved transient transfection and expression of Id2-myc fusion protein as well as an Id2-flag construct and assessment of subcellular localization with EGF or heregulin treatment. Neither altered the localization of Id2 (data not shown). This lead us to speculate that Id2 overexpression may not be capable of inducing a phenotype in the MMTV-Id2 transgenic mice due to an inability to enter the nucleus. However, immunohistochemical staining of mammary glands from these mice revealed strong nuclear staining of Id2. Thus, the lack of a phenotype in the MMTV-Id2 transgenic mice was not due to inappropriate localization of this protein.

In summary, we have found that Id2 overexpression does not induce mammary tumors on its own and it does not accelerate mammary tumorigenesis induced by chemical carcinogens or HER2/Neu. We surmise that the increased expression of Id2 that is observed in multiple forms of mammary tumorigenesis may be a result of shifting cell composition. That is, that Id2 is a marker of mammary epithelial cells that ultimately can become transformed and produce a tumor. Whether Id2 is essential for the viability of such cells remains an unanswered question that will require alternative approaches to the knock-out model we used during this study. We are currently awaiting the completion of our tumorigenesis study evaluating the impact of Id1 overexpression. As indicated above, if Id1 overexpression alters the rate of tumor formation, this would suggest that Id1 and Id2 play very distinct roles in mammary gland tumorigenesis. Identifying such roles may reveal important targets for future therapeutic intervention.

KEY RESEARCH ACCOMPLISHMENTS

Id2 knock-out mice were obtained and bred with MMTV-c-Neu mice: excessive lethality precluded further analysis.

MMTV-Id2 transgenic mice were made and overexpression of Id2 in the mammary gland was confirmed.

MMTV-Id2 transgenic mouse mammary gland whole mounts reveal no gross differences in morphology

from wild type mice.

Proliferative and apoptotic rates in MMTV-Id2 transgenic mouse mammary epithelium are indistinguishable from wild type mice.

Palpation studies revealed that MMTV-Id2 mice do not form spontaneous tumors.

Palpation studies revealed that MMTV-Id2 mice are no more susceptible to DMBA-induced carcinogenesis that wild type mice.

Palpation studies revealed that overexpression of Id2 does not accelerate HER2/Neu-induced tumorigenesis in bi-transgenic mice.

MMTV-Id1 transgenic mice were made and overexpression of Id1 in the mammary gland was confirmed.

MMTV-Id1 transgenic mouse mammary gland whole mounts reveal no gross morphological differences from wild type glands.

MMTV-Id1 mice were crossed with MMTV-c-Neu transgenic mice and animals are currently being palpated to assess differences in tumor latency.

Id2 promoter studies indicated that HER2/Neu only modestly regulates this promoter.

Id2 nuclear translocations studies revealed that HER2 or EGFR activation do not induce movement of Id2 into the nucleus.

REPORTABLE OUTCOMES

A manuscript is currently in preparation regarding the lack of phenotypes associated with Id2 overexpression. Once the Id1 studies are completed, we expect that these data will be published as well.

Meeting Abstract: Keri, R.A., Seachrist, D.D., Mosley, J.D., Milliken, E.L., and Landis, M.D., Evaluating the Roles of Id1 and Id2 in Mammary Gland Development and Carcinogenesis., Era of Hope Meeting, 2005

Presentation: Annual Meeting of the Society for the Study of Reproduction, Vancouver, Canada, 2004, “Ovary Function and Breast Disease”, Mini-symposium--Mouse Models and Gonadal Physiology—New Inroads.

CONCLUSIONS

From the studies we have described, we conclude that Id2 overeexpression does not potentiate specific mechanisms of mammary gland tumorigenesis. Specifically, we have found that Id2 overexpression also does not contribute to DMBA or HER2/Neu-induced mammary cancer. In addition, Id2 expression does not inhibit tumorigenesis, which has been speculated from some studies examining its effects of mammary epithelial cell differentiation, *in vitro*. The lack of an effect of Id2 overexpression contrasts with the increase in expression that was observed in multiple mouse models of human breast cancer. These data do not, however, indicate that Id2 expression is not necessary for tumorigenesis. Unfortunately, the anticipated model of choice for assessing this question (i.e. Id2 knock-out mice) displayed excessive lethality, precluding the most physiologically relevant approach to determining the requirement for Id2. Future studies will be aimed at determining if Id2 is necessary for tumor development using transformed breast cancer cell lines. We have also developed mice that overexpress Id1. These mice have no overt mammary gland phenotypes and we are currently assessing their tumor susceptibility. If we find that neither Id1 nor Id2 overexpression promotes mammary gland tumorigenesis, this would suggest that Id proteins may not be important targets for future study regarding breast carcinogenesis. Alternatively, if we find that Id1 overexpression alters tumorigenic rate, this would indicate that Id1 should be more extensively studied in breast cancer.

PERSONNEL SUPPORTED BY THIS PROJECT

Ruth A. Keri, Ph.D., Principal Investigator
Darcie D. Seachrist, Research Assistant
Jonathan D. Mosley, Graduate Student
Kristen Lozada, Research Assistant
John T. Poirier, Undergraduate Student
Leia Johnson, Undergraduate Student

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